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PRINCIPAL INVESTIGATOR: Kathryn D. Held, Ph.D.

CONTRACTING ORGANIZATION: Massachusetts General Hospital
Boston, Massachusetts 02114

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Kathryn D. Held, Ph.D.

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Boston, Massachusetts 02114

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This project is designed to investigate the role of apoptosis in irradiated and tamoxifen-treated breast cancer cells and to study the potential for using these manipulations to enhance tumor cell killing, thus improving radiotherapy of breast cancer. Several new observations have been made this year. First, there appears to be a lack of apoptotic-like DNA fragmentation to oligonucleosomal sized pieces in four breast cancer cell lines exposed to radiation and/or estrogen, progesterone, or tamoxifen. However, pulsed field gel electrophoresis studies suggest apoptosis is occurring in these cells, so studies using other assays for apoptosis are being initiated. A second important observation is that ER^+ cell lines may be more radiation sensitive than ER^- cells. This observation must be further studied by evaluating clonogenic radiation survival curves in additional cell lines of both types. The third important observation is that tamoxifen treatment of ER^+ cell lines appears to increase the radiation sensitivity of those cells. Although these data are at variance with the single, similar, published study, they have been reproducible in our hands in two cell lines, and are sufficiently provocative and potentially important that they must be followed up because of the obvious clinical implications.

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INTRODUCTION

It is estimated that 1 in 9 women in the US will develop breast cancer during her lifetime. Although local treatment of breast cancer, especially early breast cancer, by surgery and/or radiation therapy is quite effective, recurrence and metastases remain substantial problems limiting the cure rate of this disease. Radiation therapy plays a prominent role in the treatment of breast cancer, both as a primary and an adjuvant therapy, so increased knowledge of the mechanisms involved in ionizing radiation-induced inactivation of breast cancer cells might be expected to translate into gains in the efficacy of treating breast cancer with radiation. It has been demonstrated in other cell types that radiation can induce apoptosis, a type of cell death which is biochemically and morphologically distinct from necrosis [for general reviews on apoptosis see (1-5); for examples of studies on radiation-induced apoptosis see (6-10)]. It has also been shown that apoptosis can occur in breast tissue and breast cancer cells under normal physiological conditions and in response to hormonal manipulations (11-15). Therefore, the overall goals of this research project are to investigate the possible role of apoptosis as a mode of cell death in irradiated breast cancer cells and to study the potential for using therapeutic manipulations to enhance this apoptotic cell killing as a means of improving the efficacy of radiation therapy in the treatment of breast cancer.

The specific technical objectives of this research project are: (1) To test the hypothesis that, because breast tissue normally undergoes apoptosis in certain physiological situations, breast cancer cells are more sensitive to apoptosis induced by ionizing radiation than are cancer cells from tissues that do not normally undergo apoptosis. (2) To test the hypothesis that radiation-induced apoptosis in breast cancer cells is dependent on the proliferative status of the cells and the cell cycle phase at the time of irradiation. (3) To ascertain whether hormonal status of breast cancer cells affects the radiation sensitivity of apoptosis induction and whether hormone-induced changes in cell proliferative status alter radiation-induced apoptosis. (4) To test the hypothesis that the level of apoptosis induced by radiation in breast cancer cells can be modified by agents that modify cell survival after irradiation. (5) To ascertain whether the cellular proto-oncogene *bcl-2* plays a role in radiation-induced apoptosis and loss of clonogenicity in breast cancer cells. In all these studies, apoptosis will be determined in a quantitative assay, and the relationship between apoptosis induction and cell killing (colony formation and/or growth curves) will be determined in order to test whether apoptosis contributes significantly to long-term cell killing, i.e., whether apoptosis would be expected to contribute significantly to tumor cure.

BODY OF THE REPORT

Experimental Methods, Assumptions and Procedures

Cell lines and cell culture

The breast cancer cell lines used in the studies reported herein were MCF-7, T47D, HS578t and HTB26. Cell lines were obtained from the American Type Culture Collection (ATCC). All cells are maintained in exponential growth by twice weekly transfer in Dulbecco's minimal essential medium (DMEM) supplemented with 10% (MCF-7 and T47D) or 20% (HS578t and HTB26) fetal bovine serum, antibiotics and Hepes buffer. During the course of these studies, we found that the MCF-7 cells we were using were p53 null, rather than p53 wild-type, as they have been widely reported in the literature to be. MCF-7 cells are genetically unstable and can spontaneously lose the p53 gene in culture (P. O'Connor, personal communication to S. Powell of our Department). We have recently obtained a strain of MCF-7 cells (from Dr. H. Nagasawa, Harvard School of Public Health) that has been shown to be p53 wild type and have initiated studies with them. However, all data presented in the Tables in this report were obtained using the p53 null MCF-7 variant. Relevant characteristics of the cell lines are listed in Table I.

Table I
Relevant Characteristics of Breast Cancer Cells Used in these Studies

Cell line	ER status	p53 status[a]	bcl-2 status[a]
MCF-7 (ATCC)	positive	null	expressed
MCF-7 (HN)	positive	wild-type	ND[b]
T47D	positive	mutant	expressed
HS578t	negative	mutant	expressed (low)
HTB26	negative	ND	ND

[a] p53 status and bcl-2 status determined in our laboratory using Western blots, except for the MCF-7 (HN) variant which has been shown by Dr. Nagasawa to be functionally p53 wild-type by Westerns for increased expression of p53 and p21 after irradiation and by cell cycle analysis showing G_1 arrest.

[b] ND = not determined yet

For each experiment, cells are grown in phenol red-free DMEM containing dextran-coated charcoal treated serum (prf/dcc medium) for one week prior to the initiation of drug treatment. To initiate an experiment, cells are then replated into DMEM or prf/dcc DMEM containing an appropriate concentration of estrogen, progesterone or tamoxifen for varying lengths of time prior to irradiation or assay for cell growth, plating efficiency, etc.

Irradiation of cells

For each radiation experiment, cells are trypsinized, counted, diluted to 2 x 10^5 cells/ml, then placed in specially designed stirring, irradiation vessels. Details of our standard irradiation methods have been published (16,17). Cells are irradiated with a range of doses using a Siemens Stabilipan 2 X-ray generator operated at 250 kVp, 12 mA, dose rate about 1.4 Gy/min. Following irradiation, cells are diluted and plated in triplicate into petri dishes at cell numbers that should yield between 30 and 200 colonies per plate. The plates are incubated at 37°C for 2-6

weeks, depending on the growth rate of the cells, and colonies containing more than 50 cells are counted. Survival curves are generated according to standard protocols, and data are fit to the equation

$$SF = 1 - (1 - e^{-D/D_0})^n$$

where SF is surviving fraction for each dose D, D_0 is the reciprocal of the slope of the cell survival curve, a measure of radiation sensitivity, and n is the intercept.

Gel electrophoresis for DNA fragmentation

The appearance of a DNA "ladder" pattern, due to endonucleolytic cleavage of DNA to multimers of nucleosomal-sized pieces, on agarose gels after electrophoresis is frequently considered to be a hallmark of apoptosis (18,19). Preparation of cell lysates from drug-treated and irradiated breast cancer cells and gel electrophoresis of those lysates has been performed according to our published protocols (20,21). Human leukemia HL-60 cells exposed to 40 Gy irradiation (20) or 3.5 mM dithiothreitol (22) are used as positive controls in gels because they form readily visible DNA ladders.

Results

Radiation-induced apoptosis in breast cancer cell lines

Objective 1 of this project was to test the hypothesis that breast cancer cells are more sensitive to induction of apoptosis than are other types of cancer cells that derive from tissues that do not normally undergo apoptosis under physiological conditions. We have previously reported that, in our initial studies, none of the five breast cancer cell lines we tested showed DNA fragmentation to oligonucleosomal pieces on agarose gels after irradiation or tamoxifen treatment, although pulsed field gel electrophoresis suggested the production of large molecular weight DNA fragments occurred in some of the lines after radiation or tamoxifen treatment. The production of DNA fragments of approximately 50 or 300 kbp has been shown to be involved in apoptosis (23,24). Since there are mixed results in the literature regarding whether breast cancer cells undergo "typical" apoptosis and degrade their DNA to oligonucleosomal sized pieces (see discussion below), this year we repeated our earlier studies testing for DNA fragmentation to "ladders" in the breast cancer cell lines listed in Table I. In these additional studies, we exposed the cells to varying doses of radiation and used much longer pre-treatment times with prf/dcc (up to 7 days) and longer treatments (out to 14 days, in come cases) with a range of concentrations of estrogen, progesterone and tamoxifen. This has included treatments that cause complete growth inhibition or even cell loss, as indicated by growth curves (see next section). We have not been able to detect DNA fragmentation to oligonucleosomal pieces in any of the breast cancer cell lines after any of the drug or radiation treatments.

Growth and viability of breast cancer cells during and after hormonal manipulation

In last year's report we presented preliminary data on the doubling times over a period of a week of four breast cancer cell lines growing in the absence and presence of various concentrations of estradiol, progesterone and tamoxifen in prf/dcc medium. More complete data than those presented last year are shown here in Table II by expressing the number of cells in each culture at 7 days relative to the number plated on day 0. (Seven days is used for these assessments because that is the time used in most of the radiation experiments reported below.) In short, the new data are consistent with the preliminary data reported last year. They show that the growth of all cell lines is slowed slightly when they are cultured in phenol red-free medium,

with the effects in the ER+ MCF-7 and T47D cells being somewhat greater than in the two ER- cell lines. Addition of 0.1 - 1.0 μM estrogen to the medium has only a minimal effect on the cells, but high doses of estrogen (10 μM) decrease the growth of all four cell lines, with a particularly large effect in the T47D line, which actually loses cells during the one week growth period. In three of the cell lines, the addition of 0.1 or 1.0 μM progesterone has little effect on the cell growth, but growth of the T47D cells is slowed appreciably by low dose progesterone, and over 80% of the cells are lost in cultures exposed to 10 μM progesterone. Growth of the ER+ MCF-7 cell line is greatly decreased by the addition of 1 or 3 μM tamoxifen, there is less effect on the T47D cells, and the two ER- cell lines are unaffected. However, at 10 μM tamoxifen, MCF-7 cell numbers decrease, T47D and HS578t cell numbers remain flat for a week, and growth of HTB26 cells is slowed appreciably.

Table II
Growth of Breast Cancer Cell Lines in Estradiol, Progesterone and Tamoxifen

	Relative Cell Number[a]			
Treatment	MCF-7	T47D	HS578t	HTB26
Complete medium	4.7	5.2	3.9	11.0
Phenol red-free (prf) medium	3.5	4.3	3.8	10.5
prf + 0.1 μM estradiol	3.3	5.4	4.0	10.5
prf + 1.0 μM estradiol	3.0	3.7	4.0	10.3
prf + 10 μM estradiol	1.2	0.6	1.4	3.4
prf + 0.1 μM progesterone	3.9	2.5	3.6	11.1
prf + 1.0 μM progesterone	2.7	1.3	3.9	10.8
prf + 10 μM progesterone	0.58	0.16	2.0	3.1
prf + 1.0 μM tamoxifen	2.1	4.6	3.5	11.1
prf + 3.0 μM tamoxifen	1.9	3.9	3.4	11.6
prf + 10 μM tamoxifen	0.52	1.2	1.0	4.6

[a] Cell number is the number of cells on day 7 of treatment relative to the starting cell density on day 0. Data are averages from 1-3 separate experiments.

In last year's report we also mentioned preliminary data on the plating efficiencies of the various cell lines after drug treatments. These experiments have now been completed, and the data are presented in Table III. In these experiments, the cells were exposed to various concentrations of estradiol, progesterone and tamoxifen for 3 to 9 days after an initial week's growth in prf/dcc DMEM, then replated in drug-free, complete DMEM for colony formation. Because there were no differences in the plating efficiencies obtained for 3 to 9 day drug exposures, the data at all times have been averaged for presentation in Table III. Although each of the four breast cancer cell lines has its own characteristic plating efficiency, (e.g., HTB26 cells have a 60% PE vs. a 30% PE for HS578t cells), the clonogenic potential of all lines is completely unaffected by any of the drug treatments. In other words, although the growth of the cells in some of the drug conditions is dramatically slowed and cells are even lost from the population (Table II), those cells that are present at the time of transfer to drug-free, complete medium have the same viability (i.e., clonogenic potential) as cells that were never exposed to any treatment. This implies that the cells that are lost from the drug-treated populations (e.g., T47D cells treated with 10 μM progesterone, relative cell number of 0.16 in Table II) have detached and are lost when the medium is aspirated to replate the cells, but any attached cells have remained viable. Detachment of cells from the plates or flasks can be a characteristic of cells undergoing apoptosis (25).

Table III
Plating Efficiencies of Breast Cancer Cell Lines in Estradiol, Progesterone and Tamoxifen

	Plating Efficiency[a]			
Treatment	MCF-7	T47D	HS578t	HTB26
Complete medium	48.0 ± 24.0	55.8 ± 10.9	30.2 ± 9.8	62.5 ± 14.5
Phenol red-free (prf) medium	50.2 ± 19.5	49.9 ± 11.7	30.2 ± 6.5	61.5 ± 4.9
prf + 0.1 μM estradiol	54.3 ± 25.1	46.4 ± 14.0	27.6 ± 9.3	55.8 ± 5.0
prf + 1.0 μM estradiol	52.8 ± 24.9	47.3 ± 13.4	22.9 ± 6.5	51.4 ± 6.7
prf + 10 μM estradiol	60.9 ± 26.8	53.2 ± 15.1	28.3 ± 4.3	48.0 ± 9.3
prf + 0.1 μM progesterone	53.4 ± 17.1	38.6 ± 13.7	28.1 ± 4.4	57.4 ± 10.7
prf + 1.0 μM progesterone	54.8 ± 19.2	38.2 ± 9.0	33.2 ± 9.1	54.6 ± 15.7
prf + 10 μM progesterone	47.0 ± 12.9	59.8 ± 19.7	28.8 ± 9.7	44.2 ± 8.6
prf + 1.0 μM tamoxifen	67.1 ± 24.5	42.9 ± 14.2	31.3 ± 8.6	47.2 ± 10.1
prf + 3.0 μM tamoxifen	60.9 ± 25.5	41.8 ± 16.5	27.7 ± 7.4	51.3 ± 4.7
prf + 10 μM tamoxifen	52.7 ± 22.8	46.1 ± 9.4	28.8 ± 4.4	47.4 ± 19.4

[a] Plating efficiency is determined using a clonogenic assay on cells replated into complete DMEM after 3-9 days of the indicated treatment. Data are averages ± standard deviation from 4-8 separate experiments.

Radiation survival curves of breast cancer cell lines without and with hormonal manipulation

A major emphasis relevant to all objectives of this project was to assess the possible relationship between radiation-induced apoptosis and cell killing as measured in a clonogenic assay. Hence, in the past year, we have invested a significant amount of time on obtaining clonogenic survival curves. Data on the radiation sensitivity of the four breast cancer cell lines in the absence of any drug treatments are presented in Table IV. The data show that the radiation sensitivity of the four lines differs, with the most resistant cells, HS578t, being almost 2-fold less sensitive to radiation than the most sensitive line, MCF-7. The two ER^+ cells lines are more sensitive to radiation-induced cell killing than are the two ER^- lines, but until data are obtained on additional cell lines one can not conclude whether it is a generality that ER^+ cell lines are more radiation sensitive than ER^- ones. It is also noteworthy that the radiation sensitivity of the cells is not altered by growth in medium containing hormone-stripped serum compared to that in complete medium, although in the ER^+ cells the growth rate was slowed slightly (Table II).

Table IV
Radiation Response of Breast Cancer Cells

Cell line	D_0 in DMEM	D_0 in prf DMEM[a]
MCF-7 (p53 null)	0.89 ± 0.25 (4)[b]	1.05 ± 0.11 (4)
T47D	1.20 ± 0.17 (5)	1.16 ± 0.16 (5)
HS578t	1.88 ± 0.12 (5)	1.75 ± 0.08 (4)
HTB26	1.34 ± 0.18 (4)	1.26 ± 0.13 (4)

[a] Cells exposed to radiation after 9 - 14 days in culture in prf/dcc DMEM.

[b] D_0 values are means ± standard deviation of the number of experiments in parentheses.

As these experiments to determine the clonogenic radiation sensitivity of the various breast cancer cell lines treated with varying hormonal manipulations were being conducted and the data presented in Tables II and III were maturing, it became obvious that longer and longer drug treatments were needed prior to the irradiation in order to see significant effects on cell

growth and radiation sensitivity. In some instances, in order to see significant effects of the drugs on cell growth and radiation sensitivity, pre-treatment of cells in prf/dcc medium for as long as 7 days followed by 7 days of treatment with the drugs was needed. Hence, these experiments have been very time consuming and are still in progress. From the preliminary data obtained to date (Table V) we can make the following conclusions. In all four cell lines, 0.1 and 1.0 μM estradiol or progesterone had no effect on radiation response; hence, those data are not included in Table V. In the two ER^- cell lines, treatment for up to a week with 10 μM estradiol, progesterone or tamoxifen before irradiation also did not alter the radiation sensitivity of the cells. Hence, even though high doses of these drugs slowed cell growth significantly (Table II), the cells present at the time of irradiation were fully viable (Table III) and their radiation sensitivity was not changed. On the other hand, the ER^+ cell lines are sensitized by 10 μM estradiol or progesterone and all concentrations of tamoxifen, relative to the radiation response in prf/dcc medium without drugs.

Table V
Effect of Hormonal Manipulation on Radiation Sensitivity of Breast Cancer Cell Lines

	Enhancement Ratio[a]			
Treatment	MCF-7	T47D	HS578t	HTB26
Phenol red-free (prf) medium	1.0	1.0	1.0	1.0
prf + 10 μM estradiol	1.2	1.2	1.0	1.0
prf + 10 μM progesterone	1.3	1.5	1.0	1.0
prf + 1.0 μM tamoxifen	1.3	1.0	1.0	1.0
prf + 3.0 μM tamoxifen	1.3	1.3	1.0	1.0
prf + 10 μM tamoxifen	1.7	1.3	1.1	1.0

[a] Enhancement ratio, ER, is the ratio of the D_0 in the absence of drug to that in the presence of the drug for 7 days prior to irradiation.

Discussion

Radiation-induced apoptosis in breast cancer cell lines

We described above that, despite exhaustive testing for DNA fragmentation to oligonucleosomal sized pieces after irradiation and/or treatment with a range of doses of estrogen, tamoxifen or progesterone, we have been unable to detect this marker of apoptosis in any of the four breast cancer cell lines we have been using. This result differs from the recent demonstration that tamoxifen induced oligonucleosomal DNA fragmentation in MCF-7 cells (26). However, in a recent review, McCloskey *et al.* (27) summarize the available literature on programmed cell death in breast cancer cells treated *in vivo* or *in vitro* with estrogen withdrawal or with anti-estrogens. They noted that quite variable results have been reported by different investigators. For example, as cited by McCloskey *et al.*, following estrogen withdrawal in MCF-7 cells, one paper reports morphological and DNA oligonucleosomal degradation *in vivo*, a second paper reported *in vitro* morphological changes, but no oligonucleosomal DNA fragmentation, a third paper detected 50 and 300 kbp DNA fragments, but no morphological changes consistent with apoptosis, and a fourth paper reported neither morphological changes nor electrophoretic DNA changes on pulsed field gels or regular gels. McCloskey *et al.* point out that these variable results may reflect the existence of several phenotypic variants of MCF-7 cells. Although radiation-induced apoptosis has been demonstrated in an ever-increasing number of other cancer cell lines [recently reviewed in (10)], to our knowledge, there are no papers in the literature investigating the production of apoptosis in breast cancer cells by ionizing radiation. Hence, the findings presented here, admittedly negative to date, are new and important.

In our original proposal we had discussed the possibility that it may become necessary to use additional assays for apoptosis during the course of this study. Because an important objective of this research program is to quantitatively compare apoptosis and loss of clonogenicity, we feel it is important to use quantitative endpoints for apoptosis. We have just begun experiments using several flow cytometry based assays for apoptosis, including analysis of the "sub-G1" fraction and the TUNEL assay (28,29)and the newer method of staining for translocation of phosphatidylserine to the outer leatlet of the plasma membrane using Annexin V (30). We expect to have data from these studies shortly. These assays will also be compared with morphological changes in treated cells. It is important to use a range of assays because they measure different aspects of apoptosis and changes that occur on different time scales, e.g., Annexin V staining generally becomes visible before DNA fragmentation.

Growth and viability of breast cancer cells during and after hormonal manipulation

We report here that 10 μM estradiol, progesterone or tamoxifen slows growth or causes cell loss in all four cell lines. Tamoxifen, at lower doses, also slows the growth of the ER^+ MCF-7 cells, consistent with the dose-dependent decrease in cell proliferation shown in previous publications (31). These effects of tamoxifen on the ER^+ cell lines presumably reflect, largely, the antiestrogenic properties of tamoxifen. The effect of high dose tamoxifen on the ER^- cells may be a reflection of its activity as an inhibitor of protein kinase C (32-34), as an antagonist of calmodulin (35), or its reduction of bcl-2 expression (36). Growth inhibitory effects of high concentrations of estradiol have been demonstrated before (37).

In some cases it has been thought that changes in cell growth rates caused by hormonal manipulations reflect changes in cell cycle distributions. For example, tamoxifen treatment of MCF-7 cells has been shown to cause an increase in the proportion of G_1 phase cells , with a decrease in the percentage of S phase cells (31), and 10 μM estradiol has been reported to have the same effect, i.e., accumulation of cells in G_1 with loss in S, in MCF-7 cells, but to not alter the cell cycle in T47D cells (37). Consistent with objective 2 of our proposal, we have recently initiated cell cycle analysis using flow cytometry on the drug-treated cells in all four cell lines. We expect to have the data shortly.

Radiation survival curves of breast cancer cell lines without and with hormonal manipulation

We pointed out above that the data suggest that ER^+ cell lines may be more radiation sensitive than the ER^- lines. Few such comparisons seem to exist in the literature, although the findings are consistent with the data of Wazer *et al.* (37) that show exponential phase ER^+ MCF-7 and T47D cells to be slightly (although not statistically significant) more radiation sensitive than ER^- MDA-MB-231 cells. Additional studies with other breast cancer cell lines are needed to assess whether this trend is real.

It has been reported previously that growth inhibitory concentrations of tamoxifen result in a decrease in radiation sensitivity of MCF-7 cells (38). Our data with MCF-7 and T47D cells are not consistent with that observation, and, in fact, show significantly increased radiation sensitivity (Table V). At this time, the reason for the discrepancy in results is not clear. Perhaps, the cell cycle analysis, when completed, will give us some helpful information. Alternatively, this could reflect differences in strains of MCF-7 cells.

Recommendations

Although achievement of our objectives has been slowed by the need for longer than expected pre-treatment of cells in prf/dcc DMEM and with the hormone altering drugs, we have now worked out highly effective and reproducible cell handling conditions and are in a phase of study that will produce a great deal of data rapidly, particularly using flow cytometry for cell cycle analysis and apoptosis measurement, and obtaining the radiation clonogenic survival curves. Hence, our major emphasis in the next year will be in these three areas, and we expect to make significant progress on all the original objectives of this project in the next year.

CONCLUSIONS

In summary, the data obtained in the past year have yielded several interesting new observations. First, there appears to be a complete lack of apoptotic-like DNA fragmentation to oligonucleosomal sized pieces in four breast cancer cell lines (two ER^+ and two ER^-) exposed to radiation and/or estrogen, progesterone, or tamoxifen. This effect appears to be p53-independent, since no DNA ladders were seen in either p53 wild type or p53 null MCF-7 variants. Based on pulsed field gel studies it does appear that some of the cells undergo apoptosis, although the amount of apoptosis can not be quantitated with those gels. Therefore, studies are now being initiated using several other, newer, assays using different criteria for apoptosis.

A second important observation is that ER^+ cell lines may have a tendency to be more radiation sensitive than ER^- cells. This observation must be further studied by evaluating clonogenic radiation survival curves in additional cell lines of both types. If, indeed, there appears to be a consistent difference in the radiation sensitivity of breast cancer cells based on their ER status, that could affect ultimate decisions about clinical management of some breast cancers, i.e., whether to treat certain tumors with radiation therapy.

The third important observation from this year's data is that tamoxifen treatment of ER^+ cell lines appears to increase the radiation sensitivity of those cells. Although these data are at variance with the single, similar, limited study in the literature, they have been quite reproducible in our hands in two cell lines, and are sufficiently provocative and important that they must be continued. The clinical implications are clear. Many women with breast cancer receive both tamoxifen and radiation therapy. If some efficacy could be shown for the combination, increased cure rates may follow. The timing of the combination may prove to be important, e.g., tamoxifen before and/or during radiation might be more effective than if given after, and should be evaluated in further studies.

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